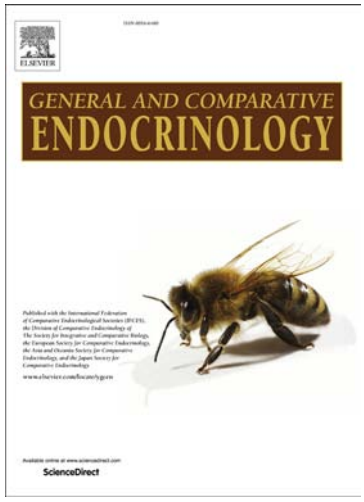


Molecular characterization of a short neuropeptide F signaling system in the tsetse fly, *Glossina morsitans morsitans*

Jelle Caers, Katleen Peymen, Matthias B. Van Hiel, Liesbeth Van Rompay, Jan Van Den Abbeele, Liliane Schoofs, Isabel Beets

PII: S0016-6480(16)30163-0
DOI: http://dx.doi.org/10.1016/j.ygcen.2016.06.005
Reference: YGCEN 12422

To appear in: *General and Comparative Endocrinology*

Received Date: 7 January 2016
Revised Date: 29 April 2016
Accepted Date: 7 June 2016

Please cite this article as: Caers, J., Peymen, K., Van Hiel, M.B., Van Rompay, L., Van Den Abbeele, J., Schoofs, L., Beets, I., Molecular characterization of a short neuropeptide F signaling system in the tsetse fly, *Glossina morsitans morsitans*, *General and Comparative Endocrinology* (2016), doi: http://dx.doi.org/10.1016/j.ygcen.2016.06.005

Title

Molecular characterization of a short neuropeptide F signaling system in the tsetse fly, *Glossina morsitans morsitans*

Authors

Jelle Caers*[a], Katleen Peymen*[a], Matthias B. Van Hiel[a], Liesbeth Van Rompay[a], Jan Van Den Abbeele[b,c], Liliane Schoofs[a], Isabel Beets[a]
* Authors contributed equally to this work

Affiliations

[a]Functional Genomics and Proteomics, Department of Biology, KU Leuven, Leuven, Belgium.
[b]Unit of Veterinary Protozoology, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium.
[c]Laboratory of Zoophysiology, Department of Physiology, University of Ghent, Krijgslaan 281, 9000 Ghent, Belgium.

E-mail addresses:

Jelle.Caers@bio.kuleuven.be
Katleen.Peymen@bio.kuleuven.be
Liesbeth.VanRompay@bio.kuleuven.be
Boris.VanHiel@bio.kuleuven.be
jvdabbeele@itg.be
Liliane.Schoofs@bio.kuleuven.be
Isabel.Beets@bio.kuleuven.be

Corresponding author:

Liliane Schoofs
Liliane.Schoofs@bio.kuleuven.be
Functional Genomics and Proteomics, Department of Biology, KU Leuven, Naamsestraat 59, 3000 Leuven, Belgium.
+32 16 37 36 97

Abstract

Neuropeptides of the short neuropeptide F (sNPF) family are widespread among arthropods and found in every sequenced insect genome so far. Prior functional studies have mainly focused on the regulatory role of sNPF in feeding behavior, although this neuropeptide family has pleiotropic effects including in the control of locomotion, osmotic homeostasis, sleep, learning and memory. Here, we set out to characterize and determine possible roles of sNPF signaling in the haematophagous tsetse fly *Glossina morsitans morsitans*, a vector of African *Trypanosoma* parasites causing human and animal African trypanosomiasis. We cloned the *G. m. morsitans* cDNA sequences of an sNPF-like receptor (Glomo-sNPFR) and precursor protein encoding four Glomo-sNPF neuropeptides. All four Glomo-sNPF peptides concentration-dependently activated Glomo-sNPFR in a cell-based calcium mobilization assay, with EC_{50} values in the nanomolar range. Gene expression profiles in adult female tsetse flies indicate that the Glomo-sNPF system is mainly restricted to the nervous system. *Glomo-snpfr* transcripts were also detected in the hindgut of adult females. In contrast to the *Drosophila* sNPF system, tsetse larvae lack expression of *Glomo-snpf* and *Glomo-snpfr* genes. While *Glomo-snpf* transcript levels are upregulated in pupae, the onset of *Glomo-snpfr* expression is delayed to adulthood. Expression profiles in adult tissues are similar to those in other insects suggesting that the tsetse sNPF system may have similar functions such as a regulatory role in feeding behavior, together with a possible involvement of sNPFR signaling in osmotic homeostasis. Our molecular data will enable further investigations into the functions of sNPF signaling in tsetse flies.

Keywords

Insect
Tsetse fly
Glossina morsitans morsitans
Neuropeptide
short neuropeptide F
G protein-coupled receptor

Abbreviations

sNPF: short neuropeptide F
sNPFR: short neuropeptide F receptor
GPCR: G protein-coupled receptor
CHO: Chinese hamster ovary
NPF: neuropeptide F

1. Introduction

Tsetse flies (genus *Glossina*) are the transmitters of African trypanosomes throughout sub-Saharan Africa, thereby causing human and animal African trypanosomiasis with impact on human health and local economy. One of the best trypanosomiasis control strategies has proven to be the reduction of tsetse fly populations (Attardo et al., 2010). The success of this vector control resides in the low reproduction potential of tsetse flies stemming from their viviparous reproductive strategy, which is rather unique in insects. Female tsetse flies can generate only 8 to 12 individuals during their lifetime (Tobe and Langley, 1978), which are nourished during larvagenesis via intrauterine milk secretions from the maternal accessory gland (Denlinger and Ma, 1974). Similarly to tsetse reproduction physiology, their feeding behavior is also rather unique because both males and females are obligate blood feeders and rely exclusively on proline as major energy source (Bursell, 1977). However, little is known about the regulation of this blood feeding behavior (Lehane, 2005). Studying the involved signaling systems can point towards interesting targets that may decrease fly fitness and lead to the development of new vector control strategies.

Neuropeptide systems are essential signaling systems that are well conserved during evolution and engaged in many physiological functions, including feeding behavior. Amongst these, the short neuropeptide F (sNPF) system has been widely linked to feeding behavior. So far, it has been exclusively identified in Arthropoda, wherein sNPFs are characterized by an xPxLRLRFamide consensus sequence (Nässel and Wegener, 2011). From transcript analyses, peptide administration and proteomics studies, it has become evident that sNPFs have opposing effects on feeding depending on the insect species. While it promotes food intake in some species, such as *Drosophila*, it negatively correlates with food intake in other species such as the silk moth *Bombyx mori* (Nagata et al., 2012, reviewed by Spit et al., 2012).

Besides a well-described role in the regulation of feeding behavior, sNPF has been implicated in other physiological processes. In *Drosophila*, sNPF signaling is involved in locomotion (Kahsai et al., 2010b), metabolism and stress resistance (Kahsai et al. 2010; Kapan et al. 2012). In addition, sNPFs are thought to regulate hormone release (Nässel et al., 2008), circadian rhythm (Johard et al., 2009), osmotic homeostasis (Kahsai et al. 2010) and learning and memory (Dillen et al., 2015; Johard et al., 2008). In *B. mori*, sNPF has been associated with suppression of juvenile hormone synthesis (Kaneko and Hiruma, 2014; Yamanaka et al., 2008).

With the publication of its genome in 2014, we identified several neuropeptide systems in the tsetse fly *Glossina morsitans morsitans*, including an sNPF system (International *Glossina* Genome Initiative, 2014). *In silico* studies predicted a single *G. m. morsitans snpf* (*Glomo-snpf)* gene with four putative Glomo-sNPF peptides (International *Glossina* Genome Initiative, 2014). This is in accordance with findings in other insect species in which only one *snpf* gene is present encoding one to five

sNPFs, with the exception of *Aedes aegypti* which has two *snpf* genes (Predel et al., 2010). In addition, the *Glossina* Genome Initiative predicted a *G. m. morsitans snpfr* (*Glomo-snpfr*) gene that encodes a single Glomo-sNPFR of the rhodopsin G-protein coupled receptor (GPCR) family. Besides sNPF, over 30 other neuropeptides are conserved in tsetse flies including a single gene for neuropeptide F (Glomo-NPF) and the neuropeptide F receptor Glomo-NPFR (International *Glossina* Genome Initiative, 2014)

Here we further characterized the predicted sNPF system of the tsetse fly and studied the activation of Glomo-sNPFR by its neuropeptide ligands as well as the spatiotemporal expression of *Glomo-snpf* and *Glomo-snpfr* genes in female tsetse flies.

2. Materials and methods

2.1 Animal rearing conditions

G. m. morsitans flies were obtained from the Institute of Tropical Medicine in Antwerp (Elsen et al., 1993) and reared at 26°C and 65% relative humidity. Flies were fed three times a week using an artificial membrane with defibrinated and gamma-irradiated bovine blood.

2.2 Cloning of *Glomo-snpf* and *Glomo-snpfr* cDNAs

The open reading frames (ORFs) encoding the predicted *G. m. morsitans* sNPFR (*Glomo-snpfr*. Vectorbase ID: (GMOY006636) and sNPF (*Glomo-snpf*. Vectorbase ID: (GMOY012142) precursor were amplified from female *G. m. morsitans* cDNA using Advantage 2 Polymerase (Clontech) and the following primers (Sigma-Aldrich) for *Glomo-snpfr*: Fw 5'- CACCATGCCCAACTTCAATCTCACCAAGAC-3' and Rev 5'-TCAGTTGCAGCTTTGTTTGTTTCGCTC-3' and *Glomo-snpf*: Fw 5'-CATGCATTTTCGCAGTCG-3' and Rev 5'-CATTCGTGCAGCGCATTTAG-3'. The *Glomo-snpfr* forward primer was designed with a partial Kozak sequence preceding the authentic start codon to optimize initiation of translation in mammalian cells which were used in the calcium mobilization assay (Kozak, 1987). PCR products were purified and subsequently extended with a single 3' A-overhang by making use of dATP and *Taq* polymerase before cloning to enable T/A cloning into the pcDNA3.1/V5-His-TOPO®TA expression vector (*Glomo-snpfr*) or the pCR4-TOPO® TA sequencing vector (*Glomo-snpf*) (Invitrogen). Cloned *Glomo-snpfr* and *Glomo-snpf* constructs were transformed into One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen) and plasmids were isolated with the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). Cloned cDNA sequences were determined on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) using the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems).

2.3 Sequence analyses of Glomo-sNPFR and Glomo-sNPF precursor

The protein sequence of the cloned Glomo-sNPFR ORF was analyzed for the presence of putative transmembrane regions with the software programs PSIPRED and MEMSAT3 (bioinf.cs.ucl.ac.uk/psipred) (Jones, 2007). Phosphorylation, glycosylation and palmytoylation sites were respectively predicted with NetPhos 2.0, NetNGlyc 1.0 and GSS-PALM 4.0 (www.cbs.dtu.dk/services/NetPhos, www.cbs.dtu.dk/services/NetNGlyc and csspalm.biocuckoo.org/online.php) (Blom et al., 1999; Gupta and Brunak, 2002; Ren et al., 2008). Protein sequence alignment of Glomo-sNPFR with other dipteran sNPFRs was performed with Clustal Omega (www.clustal.org) (Sievers et al., 2011) and included *D. melanogaster* sNPFR (Drome-sNPFR: CG7395) (Mertens et al., 2002), *Musca domestica* sNPFR (Musdo-sNPFR: MDOA011011), *Anopheles gambiae* sNPFR (Anoga-sNPFR: AGAP012378) (Garczynski et al., 2007), and *Aedes aegypti* sNPFR (Aedae-sNPFR: AGX84996.1). The protein sequence of the cloned sNPF precursor was analyzed for the presence of an N-terminal signal peptide using SignalP 4.1 (www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011).

2.4 Spatial and temporal transcript profiles of *Glomo-snpf* and *Glomo-snpfr*

Transcript profiles of *Glomo-snpf* and *Glomo-snpfr* were determined by real-time quantitative PCR (RTqPCR) in different tissues of female tsetse flies and at different developmental stages for which sex could not be distinguished. Tissues were dissected under a binocular microscope, collected in ice-cold phosphate buffered saline (PBS) (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.76 mM; pH 7.4) and immediately frozen in liquid nitrogen. Three biological repeats were assembled for each tissue. For each biological repeat, the following tissues were collected: a single female head, thorax and abdomen; an 18-day-old pupal head, thorax and abdomen; a complete 18-day-old pupa; an 11-day-old pupa; and first, second and third larval instars. Additionally, tissues from five flies were pooled in one biological repeat for: the female reproductive system including the uterus and ovaries; the flight muscles; the fat body including the milk gland; the anterior midgut; the posterior midgut; the Malpighian tubules; and the entire brain including the optical lobes, antennal lobes and suboesophageal ganglion. For the thoracic ganglion, corpora cardiaca and corpora allata, salivary glands, and hindgut, tissues from 10 females were pooled for one biological repeat. The dissected tissues were subsequently transferred to MagNA Lyser Green Beads tubes (Roche) and homogenized with the MagNa Lyser® (Roche). Total RNA isolation was performed using the RNeasy® Lipid Tissue Mini Kit (Qiagen) in combination with a DNase digestion (RNase-free DNase Set, Qiagen). The PrimeScript™ RT Reagent Kit (Perfect Real Time; TaKaRa Bio Inc.) was used to generate cDNA that was diluted 10-fold. For every tissue sample, a negative control sample was generated to control for the presence of unwanted genomic DNA in which reverse transcriptase was omitted from the cDNA synthesis protocol. The Fast SYBR® Green Master Mix (Applied Biosystems) was used for all RTqPCR experiments. Reference genes for normalization of the RTqPCR results were determined with the geNorm applet of

qbase$^+$ 2.4 (Biogazelle; see Supplementary data 1 for primer sequences, thermocycle details and reaction efficiencies) (Hellemans et al., 2007; Vandesompele et al., 2002). *cyp33*, *actin C* and *β-tub* genes were found to be most stably expressed in the total sample set. In addition to three biological repeats, three technical replicates were run for each individual sample. Every primer set was checked for potential master mix contamination by adding a no template control. Data analysis was performed according to the $\Delta\Delta C_T$ method with qbase$^+$ (Livak and Schmittgen, 2001).

2.5 Cell culture and transient transfections

Functional activation studies of Glomo-sNPFR were performed in Chinese hamster ovary cells (CHO-K1) that stably overexpress an apoaequorin calcium indicator targeted to mitochondria (mtAeq), a promiscuous human $G\alpha_{16}$ subunit, and a zeocin resistance gene (CHO/mtAeq/$G\alpha_{16}$) (Stables et al., 1997). The promiscuous $G\alpha_{16}$ subunit directs the intracellular signaling pathway of most GPCRs to the activation of phospholipase C and subsequent release of calcium from intracellular stores (Brough and Shah, 2009; Offermanns and Simon, 1995). The CHO/mtAeq/$G\alpha_{16}$ cell line was cultured in monolayer in Dulbecco's Modified Eagles Medium nutrient mixture F12-Ham (DMEM/F12; Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 IU/ml of penicillin/streptomycin (P/S, Invitrogen), 250 µg/ml zeocin (Invitrogen) and 2.5 µg/ml fungizone (Amphotericin B; Invitrogen). Downstream intracellular signaling of the Glomo-sNPFR was investigated using CHO/mtAeq cells that do not express a $G\alpha_{16}$ subunit. These cells were cultured in DMEM/F12 supplemented with 10% FBS, 100 IU/ml P/S, 2.5 µg/ml fungizone and 5 µg/ml puromycin (Invitrogen). Both cell lines were incubated at 37°C and 5% CO_2 in a humidified atmosphere and were passaged every three days (1:10). When cells reached 60-80% confluence, they were transfected with pcDNA3.1-*Glomo-snpfr* or empty pcDNA3.1 vector using Lipofectamine LTX (Invitrogen). After 16 to 18 hours at 37°C and 5% CO_2, cells were supplemented with fresh cell medium and incubated overnight (37°C, 5% CO_2) before the calcium mobilization assay was performed.

2.6 *In vitro* calcium mobilization assay

Calcium responses of CHO cells transfected with pcDNA3.1-*Glomo-snpfr* or empty pcDNA3.1 vector were measured with an aequorin-based calcium mobilization assay. Transfected cells were first detached with PBS containing 0.2 % EDTA and collected in 10 mL complete DMEM/F12 medium. Cell density was determined with the NucleoCounter (Chemometec), and cells were resuspended in bovine serum albumin (BSA) medium (DMEM/F12 without phenol red with L-glutamine and 15 mM HEPES, Gibco, supplemented with 0.1% BSA, Sigma-Aldrich) to a density of 5 x 10^6 cells per mL. Next, cells were loaded with 5 µM coelenterazine h (Invitrogen) and incubated for 4 hours at room temperature to reconstitute the aequorin calcium indicator. 96-well compound plates were prepared containing a concentration series of the different Glomo-sNPF peptides. The *G. m. morsitans* neuropeptide F (Glomo-NPF),

which shares the same C-terminal RxRFamide with Glomo-sNPF neuropeptides, was also tested for its ability to activate Glomo-sNPFR. Additionally, wells with only BSA medium were set as negative controls. ATP (1 µM) was included as a positive control, which activates an endogenous CHO cell receptor. Compound plates were placed in the Mithras LB940 luminometer (Berthold Technologies). Cells were then added to the wells by the luminometer device at a density of 25,000 cells/ well, and luminescence was measured for 30 s at a wavelength of 469 nm. After 30 s, 0.1% triton X-100 was added to lyse the cells, resulting in a maximal calcium response that was measured for 10 s. To exclude activation of endogenous CHO receptors by the Glomo-sNPF peptides, cells transfected with an empty pcDNA3.1 construct were also tested. Three independent experiments each containing three replicates of the peptide concentration series were conducted. GraphPad Prism 5 was used to analyze data and construct concentration response curves (nonlinear regression analysis with a sigmoidal concentration-response equation).

2.7 Peptides

Glomo-sNPF-1 (AQRSPSLRLRFamide), Glomo-sNPF-2 (SPSLRLRFamide), Glomo-sNPF-3 (PQRLRFamide), Glomo-sNPF-4 (PARLRFamide) and Glomo-NPF (NTRPTRNDEIANIEEALQYLQELENFYDGKARIRFamide) were synthesized by GL Biochem Ltd. (Shangai). All peptides were purified by reversed-phase high performance liquid chromatography (HPLC), and their sequence was verified with a matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI TOF/TOF, Ultraflex II Bruker Daltonics) mass spectrometer.

3. Results

3.1 Cloning and sequence analyses of G. *m. morsitans snpfr* and *snpf* cDNAs

We previously predicted through gene annotation and BLAST searches the presence of a *Glomo-snpf* and *Glomo-snpfr* gene in the *G. m. morsitans* genome (International *Glossina* Genome Initiative, 2014). Using gene-specific primers based on the predicted sequences, we isolated *Glomo-snpf* and *Glomo-snpfr* cDNAs from female *G. m. morsitans*. Translated protein sequences of the cloned cDNAs were identical to the predicted protein sequences of Glomo-sNPFR and Glomo-sNPF. The Glomo-sNPF precursor sequence encodes a 25 amino acid long signal peptide and four putative sNPF neuropeptides (Figure 1), of which two peptides share the [xPxLRLRFamide] consensus motif of the sNPF neuropeptide family (Nässel and Wegener, 2011). Protein sequence alignments of Glomo-sNPFR with other dipteran sNPFRs demonstrate a high overall sequence identity and similarity, with the highest conservation in the transmembrane domains (supplementary data 2). For example, Glomo-sNPFR shares 66% sequence identity with Musdo-sNPFR, 64% with Anoga-sNPFR, 63% with Drome-sNPFR, and 59% with Aedae-sNPFR. In addition, the Glomo-sNPFR sequence harbors typical motifs of the rhodopsin-like GPCR family

and multiple putative phosphorylation, palmitoylation and glycosylation sites (Figure 2).

3.2 Spatial and temporal transcript profiles of *Glomo-snpfr* and *Glomo-snpf*

We investigated temporal and spatial distributions of *Glomo-snpfr* and *Glomo-snpf* transcripts by RTqPCR using RNA samples from different tissues of adult female tsetse flies and from different developmental stages. For both *Glomo-snpf* and *Glomo-snpfr* genes, highest transcript levels were found in the corpora cardiaca and corpora allata of adult female flies (Figure 3A, B). In addition, *Glomo-snpfr* transcripts were expressed at moderate levels in the thoracic ganglion, hindgut, brain, and head samples. Other examined tissues showed no or very low *Glomo-snpfr* expression. Absence of *Glomo-snpfr* expression was also seen during larval and pupal development (Figure 3A). Similarly to the results for *Glomo-snpfr*, *Glomo-snpf* expression was observed in the thoracic ganglion, brain, and head samples of adult females, and was absent in the different larval instars. In contrast, *Glomo-snpf* expression was noticeable in 11-day-old pupae and in the heads of 18-day-old pupae (Figure 3B).

3.3 Functional activation of Glomo-sNPFR by Glomo-sNPF neuropeptides

To determine whether Glomo-sNPF neuropeptides can functionally activate Glomo-sNPFR, we used an *in vitro* calcium mobilization assay (Beets et al., 2011; Brough and Shah, 2009). .All four putative Glomo-sNPF peptides concentration-dependently activated the heterologously expressed Glomo-sNPFR. All peptide-receptor couples displayed half-maximal effective concentrations (EC_{50}) in the nanomolar range: 0.72 ± 1.01 nM (95% confidence interval) for Glomo-sNPF-1, 1.61 ± 0.52 nM for Glomo-sNPF-2, 2.17 ± 0.65 nM for *Glomo*-sNPF-3 and 2.48 ± 1.02 nM for Glomo-sNPF-4 (Figure 4). In contrast, cells transfected with an empty expression vector and challenged with the sNPF peptides yielded no calcium responses (data not shown). As a control, we challenged the Glomo-sNPFR with Glomo-NPF that shares the C-terminal end (-RxRFamide) of Glomo-sNPFs. Glomo-NPF was unable to activate Glomo-sNPFR (data not shown), consistent with reports in other insects (Dillen et al., 2013).

To investigate intracellular signaling of Glomo-sNPFR, we measured calcium responses of this receptor in CHO cells lacking the promiscuous $G\alpha_{16}$ subunit. Activation of Glomo-sNPFR by all four *Glomo*-sNPF neuropeptides induced strong calcium responses, indicating that Glomo-sNPFR can couple to endogenous $G\alpha_q$-type subunits in CHO/mtAeq cells. For all peptides, EC_{50} values were similar to those observed in the presence of the $G\alpha_{16}$ protein (table 1): 2.21 ± 1.87 for Glomo-sNPF-1, 3.42 ± 2.99 for Glomo-sNPF-2, 2.67 ± 2.27 for Glomo-sNPF-3 and 11.83 ± 9.25 for Glomo-sNPF-4.

4. Discussion

The present study extended the insect sNPF research to the tsetse fly *G. m. morsitans*. We amplified the *G. m. morsitans snpf* and *snpfr* cDNA sequences and showed their corresponding protein sequences are identical to Glomo-sNPF and Glomo-sNPFR sequences predicted *in silico* (International *Glossina* Genome Initiative, 2014). The Glomo-sNPF precursor consists of 300 amino acids, including a 25 amino acid N-terminal signal peptide and four putative sNPF peptides. Of these four peptides, the biosynthesis of Glomo-sNPF-1 and Glomo-sNPF-2 has been confirmed *in vivo* by mass spectrometric analysis of tsetse extracts (Caers et al., 2015). Each of the four Glomo-sNPFs carries a C-terminal glycine residue for amidation and is flanked by mono- or dibasic cleaving sites. Glomo-sNPF-1 (AQRSPSLRLRFamide) and Glomo-sNPF-2 (SPSLRLRFamide) answer to the sNPF consensus sequence xPxLRLRFamide (Nässel and Wegener, 2011), and are identical to two *D. melanogaster* sNPF peptides (Drome-sNPF-1 and Drome-sNPF-$1_{4\text{-}11}$) (Nässel and Wegener, 2011). In contrast, Glomo-sNPF-3 (PQRLRFamide) and Glomo-sNPF-4 (PARLRFamide) lack the N-terminal leucine residue of the consensus sequence. The *Glomo-snpf* gene contains two introns (between nucleotides encoding for S^{32}/A^{33}, and for K^{285} /K^{286}) at positions similar to the *D. melanogaster snpf* gene (Q^{36}/G^{37} and K^{266}/H^{267}), although the latter contains a third intron sequence (Q^{189}/I^{190}) (Flybase - CG13968). The presence of two identical sNPFs and two relatively well conserved intron positions corroborate the annotation of the *G. m. morsitans snpf* gene.

All arthropod sNPFRs identified so far belong to the superfamily of rhodopsin-like GPCRs (Chen and Pietrantonio, 2006; Dillen et al., 2013; Feng et al., 2003; Garczynski et al., 2007; Hauser et al., 2008; Mertens et al., 2002; Veenstra et al., 2012). Likewise, the Glomo-sNPFR sequence harbors typical rhodopsin-like amino acid patterns in its seven transmembrane domains: GN in helix 1, NLX_3DX_8P in helix 2, $SX_3LX_2IX_2DRY$ in helix 3, WX_8P in helix 4, FX_2PX_7Y in helix 5, FX_3WXP in helix 6 and NPX_2YX_6F in helix 7 (Costanzi, 2012). The presence of multiple putative sites for phosphorylation (14), glycosylation (6) and palmitoylation (2) suggests post-translational modifications can regulate Glomo-sNPFR signaling. Protein sequence alignments of *Glomo*-sNPFR with other Dipteran sNPFRs revealed that their membrane spanning α-helices as well as the length and residues of intra- and extracellular loops are strongly conserved. One notable exception is the length of the second extracellular loop, which is clearly longer in fly species (*G. m. morsitans*, *D. melanogaster* and *M. domestica*) than in mosquito species (*A. gambiae,* and *A. aegypti*). In insects from other orders, such as *Solenopsis invicta* (Hymenoptera) or *Schistocerca gregaria* (Orthoptera), the length of this loop is comparable with those from mosquito species (Dillen et al., 2013). It remains unclear how this variable loop length may affect the functioning of sNPFRs, but possible effects include altered G-protein coupling or post-translational regulation.

Using cell-based functional studies we showed that Glomo-sNPFR is dose-dependently activated by Glomo-sNPF neuropeptides. All four Glomo-sNPF

neuropeptides activate Glomo-sNPFR with equal potency and EC_{50} values in the nanomolar range, suggesting that the identified Glomo-sNPF peptides are likely cognate ligands of the tsetse fly sNPFR. In contrast, Glomo-NPF, which shares the same C-terminal end (-RxRFamide), did not activate Glomo-sNPFR. This supports former studies in *Drosophila* showing that the -RxRFamide motif alone is insufficient for potent receptor activation (Feng et al., 2003; Mertens et al., 2002) and corroborates an only distant evolutionary relationship between NPFRs and sNPFRs (Nässel and Wegener, 2011).

Further analysis into second messenger signaling in cells without the promiscuous $G\alpha_{16}$ subunit indicated that activation of Glomo-sNPFR with Glomo-sNPFs still results in an increase in intracellular calcium with a nanomolar EC_{50} value. This endogenous coupling of sNPFRs with a $G\alpha_q$ subunit in CHO/mtAeq cells has been observed for other insect sNPFRs as well, such as *S. gregaria* (Dillen et al., 2013). Other downstream signaling pathways may, however, also be involved in Glomo-sNPFR signaling. For example, activation of *S. gregaria* and *A. gambiae* sNPFRs was found to inhibit adenylyl cyclase activity (Dillen et al., 2013; Garczynski et al., 2007). In contrast, activation of the *D. melanogaster* sNPFR by sNPF peptides has been shown to activate adenylyl cyclase through $G\alpha_s$ (Chen et al., 2013; Hong et al., 2012).

The spatial expression of *Glomo-snpf* and *Glomo-snpfr* transcripts in adult females is mainly restricted to the nervous system. *Glomo-snpfr* expression was not detected in larvae and pupae at the time points investigated. *Glomo-snpf* was likewise undetectable in larval instars, but did appear in 11- and 18-day pupae. This absence of expression in larvae is in contrast to *Drosophila*, where a large number of neurons of the central nervous system in both larvae and adults expresses *snpf* (Carlsson et al., 2013; Johard et al., 2008; Lee et al., 2004; Nässel et al., 2008). One possible explanation for this difference lies in the feeding behaviour of tsetse larvae compared to *Drosophila* larvae. Tsetse larvae are fed in utero on milk provided by the mother. After larviposition, the larvae immediately burrow into the soil and pupariate after one or two hours. Consequently, whereas *Drosophila* larvae forage, tsetse larvae do not. Since sNPF has been implicated in feeding initiation (Nagata et al., 2011) and food-seeking behavior (Carlsson et al., 2013; Root et al., 2011), this could explain the absence of sNPF expression in tsetse larvae.

For both *Glomo-snpf* and *Glomo-snpfr*, the highest expression levels were detected in the corpora cardiaca-corpora allata, while moderate levels were found in the thoracic ganglion, brain and head samples. Mass spectrometric studies confirmed the expression of Glomo-sNPF-1 in the brain and thoracic ganglion, and the presence of Glomo-sNPF-2 in the brain, thoracic ganglion and corpora cardiaca (Caers et al., 2015). The spatial transcript distributions *of Glomo-snpf and Glomo-snpfr* corroborate with the generally restricted expression of sNPF signaling components in the central nervous system of insects. This expression is linked to their neuromodulatory and hormonal functions in a variety of physiological processes, but with a primary function

in feeding behavior (Chen and Pietrantonio, 2006; Chintapalli et al., 2007; Dillen et al., 2016, 2014; Feng et al., 2003; Garczynski et al., 2007; Kahsai et al., 2012, 2010a, 2010b; Lu and Pietrantonio, 2011; Mertens et al., 2002; Mikani et al., 2012; Nagata et al., 2012). Whereas sNPF has been widely indicated in feeding behaviour, opposing effects have been reported. sNPF stimulates feeding and starvation-induced food-searching behaviour in *Drosophila*: gain- and loss-of-function mutations of the *snpf* gene promote or suppress food intake, respectively, and *snpfr* expression increases during starvation (Lee et al., 2004; Root et al., 2011). Expression of *snpfr* in the brain is also upregulated by food deprivation in the honey bee (Ament et al., 2011). An opposite effect on feeding has been demonstrated in, for instance, *B. mori* and *S. gregaria,* where *snpfr* transcript levels decrease in the central nervous system amongst other tissues during food deprivation (Dillen et al., 2013; Nagata et al., 2012, 2011). Since *Glomo-snpf* and *Glomo-snpfr* expression in adults was primarily detected in the brain, sNPF may regulate feeding behaviour in tsetse flies as well. However, functional studies are necessary to determine its involvement and whether it has stimulatory or inhibitory effects on food intake.

The presence of sNPF and sNPFR in the corpora cardiaca-corpora allata has previously been associated with the inhibition of juvenile hormone production (Kaneko and Hiruma, 2014; Yamanaka et al., 2008). In *B. mori*, allatotropin is thought to inhibit the expression or release of sNPF from the corpora cardiaca, which prevents sNPFR activation in the corpora allata and inhibits the corresponding allatostatic activity of sNPF (Yamanaka et al., 2008). No differentiation was made between the corpora cardiaca and corpora allata in the tsetse samples, hence it remains unclear if *snpf* and *snpfr* expression is restricted to the corpora cardiaca and/or the corpora allata, respectively. Neither the allatotropin prepropeptide nor its receptor orthologues were identified in the *G. m. morsitans* genome, making it uncertain if a similar role for sNPF signaling in juvenile hormone synthesis holds for *Glossina*. This does not rule out a putative role of Glomo-sNPF as a more general regulator of hormone release. In *Drosophila* for instance, expression of sNPF in protocerebral neurons projecting to the corpora cardiaca has been linked to a co-regulatory function of sNPF on adipokinetic hormone release or on *Drosophila* insulin-like peptide release in the corpora cardiaca (Lee et al., 2009, 2008). sNPF expression in the corpora cardiaca was also previously described in *Periplanata americana* and its direct release into the circulation function in suppressing digestive activity during starvation (Mikani et al., 2015, 2012).

Besides expression in the nervous system, moderate levels of *Glomo-snpfr* were detected in the hindgut and/or its associated tissues. Expression of *snpfr* transcripts in this region was also reported in *S. invicta* (Chen and Pietrantonio, 2006) and may point to a regulatory function in diuresis to control osmotic homeostasis. In tsetse flies, feeding and subsequent excretion of water from the blood meal are closely intertwined, however, future research is required to clarify the role of sNPF in either

of these processes. Overall, our molecular data provide a foundation for further characterization and validation of this signaling system *in vivo*.

5. Acknowledgements

The work was funded by grants from the Research Foundation Flanders (FWO-Flanders, G069713N), the European Research Council (ERC grant 282312), the InterUniversity Attraction Pole program P7/41 and the ITM-SOFI funding. Isabel Beets (postdoctoral fellowship) and Katleen Peymen (doctoral fellowship) are supported by the FWO.

6. References

Ament, S.A., Velarde, R.A., Kolodkin, M.H., Moyse, D., Robinson, G.E., 2011. Neuropeptide Y-like signalling and nutritionally mediated gene expression and behaviour in the honey bee. Insect Mol. Biol. 20, 335–345. doi:10.1111/j.1365-2583.2011.01068.x

Attardo, G., Ribeiro, J., Wu, Y., Berriman, M., Aksoy, S., 2010. Transcriptome analysis of reproductive tissue and intrauterine developmental stages of the tsetse fly (Glossina morsitans morsitans). BMC Genomics 11, 1–10. doi:10.1186/1471-2164-11-160

Beets, I., Lindemans, M., Janssen, T., Verleyen, P., 2011. Deorphanizing G Protein-Coupled Receptors by a Calcium Mobilization Assay, in: Merighi, A. (Ed.), Neuropeptides, Methods in Molecular Biology. Humana Press, pp. 377–391.

Blom, N., Gammeltoft, S., Brunak, S., 1999. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites1. J. Mol. Biol. 294, 1351–1362. doi:10.1006/jmbi.1999.3310

Brough, S., Shah, P., 2009. Use of Aequorin for G protein-Coupled Receptor Hit Identification and Compound Profiling, in: Leifert, W.R. (Ed.), G Protein-Coupled Receptors in Drug Discovery, Methods in Molecular Biology. Humana Press, pp. 181–198.

Bursell, E., 1977. Synthesis of proline by fat body of the tsetse fly (Glossina morsitans): metabolic pathways. Insect Biochem. 7, 427–434. doi:10.1016/S0020-1790(77)90068-3

Caers, J., Boonen, K., Van Den Abbeele, J., Van Rompay, L., Schoofs, L., Van Hiel, M., 2015. Peptidomics of Neuropeptidergic Tissues of the Tsetse Fly *Glossina morsitans morsitans*. J. Am. Soc. Mass Spectrom. 26, 2024–2038. doi:10.1007/s13361-015-1248-1

Carlsson, M.A., Enell, L.E., Nässel, D.R., 2013. Distribution of short neuropeptide F and its receptor in neuronal circuits related to feeding in larval *Drosophila*. Cell Tissue Res. 353, 511–523. doi:10.1007/s00441-013-1660-4

Chen, M.-E., Pietrantonio, P.V., 2006. The short neuropeptide F-like receptor from the red imported fire ant, Solenopsis invicta Buren (Hymenoptera: Formicidae). Arch. Insect Biochem. Physiol. 61, 195–208. doi:10.1002/arch.20103

484 Chen, W., Shi, W., Li, L., Zheng, Z., Li, T., Bai, W., Zhao, Z., 2013. Regulation of
485 sleep by the short neuropeptide F (sNPF) in *Drosophila melanogaster*. Insect
486 Biochem. Mol. Biol. 43, 809–819. doi:10.1016/j.ibmb.2013.06.003
487 Chintapalli, V.R., Wang, J., Dow, J.A.T., 2007. Using FlyAtlas to identify better
488 *Drosophila melanogaster* models of human disease. Nat Genet 39, 715–720.
489 doi:10.1038/ng2049
490 Costanzi, S., 2012. Homology Modeling of Class A G Protein-Coupled Receptors.
491 Methods Mol. Biol. Clifton Nj 857, 259–279. doi:10.1007/978-1-61779-588-
492 6_11
493 Denlinger, D.L., Ma, W.-C., 1974. Dynamics of the pregnancy cycle in the tsetse
494 *Glossina morsitans*. J. Insect Physiol. 20, 1015–1026. doi:10.1016/0022-
495 1910(74)90143-7
496 Dillen, S., Chen, Z., Broeck, V.J., 2015. Assaying Visual Memory in the Desert
497 Locust. Insects 6. doi:10.3390/insects6020409
498 Dillen, S., Chen, Z., Vanden Broeck, J., 2016. Nutrient-dependent control of short
499 neuropeptide F transcript levels via components of the insulin/IGF signaling
500 pathway in the desert locust, *Schistocerca gregaria*. Insect Biochem. Mol. Biol.
501 68, 64–70. doi:10.1016/j.ibmb.2015.11.007
502 Dillen, S., Verdonck, R., Zels, S., Van Wielendaele, P., Vanden Broeck, J., 2014.
503 Identification of the short neuropeptide F precursor in the desert locust:
504 Evidence for an inhibitory role of sNPF in the control of feeding. Invertebr.
505 Neuropept. XIV 53, 134–139. doi:10.1016/j.peptides.2013.09.018
506 Dillen, S., Zels, S., Verlinden, H., Spit, J., Van Wielendaele, P., Vanden Broeck, J.,
507 2013. Functional Characterization of the Short Neuropeptide F Receptor in the
508 Desert Locust, *Schistocerca gregaria.* PLoS ONE 8, e53604.
509 doi:10.1371/journal.pone.0053604
510 Elsen, P., Van Hees, J., De Lil, E., 1993. L'historique et les conditions d'élevage des
511 lignèes de glossines (Diptera Glossinidae) maintenues à l'Institut de Médecine
512 tropicale Prince Léopold d'Anvers. J. Afr. Zool. 107, 439 – 449.
513 Feng, G., Reale, V., Chatwin, H., Kennedy, K., Venard, R., Ericsson, C., Yu, K.,
514 Evans, P.D., Hall, L.M., 2003. Functional characterization of a neuropeptide F-
515 like receptor from *Drosophila melanogaster*. Eur. J. Neurosci. 18, 227–238.
516 doi:10.1046/j.1460-9568.2003.02719.x
517 Garczynski, S.F., Crim, J.W., Brown, M.R., 2007. Characterization and expression of
518 the short neuropeptide F receptor in the African malaria mosquito, *Anopheles*
519 *gambiae*. Invertebr. Neuropept. VIIInvertebrate Neuropept. Conf. 2006 28,
520 109–118. doi:10.1016/j.peptides.2006.09.019
521 Gupta, R., Brunak, S., 2002. Prediction of glycosylation across the human proteome
522 and the correlation to protein function. Presented at the Pacific Symposium on
523 Biocomputing, World Scientific, pp. 310–322.
524 Hauser, F., Cazzamali, G., Williamson, M., Park, Y., Li, B., Tanaka, Y., Predel, R.,
525 Neupert, S., Schachtner, J., Verleyen, P., Grimmelikhuijzen, C.J.P., 2008. A
526 genome-wide inventory of neurohormone GPCRs in the red flour beetle
527 *Tribolium castaneum*. Front. Neuroendocrinol. 29, 142–165.
528 doi:10.1016/j.yfrne.2007.10.003
529 Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007.
530 qBase relative quantification framework and software for management and
531 automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19–
532 R19. doi:10.1186/gb-2007-8-2-r19

Hong, S.-H., Lee, K.-S., Kwak, S.-J., Kim, A.-K., Bai, H., Jung, M.-S., Kwon, O.-Y., Song, W.-J., Tatar, M., Yu, K., 2012. Minibrain/Dyrk1a Regulates Food Intake through the Sir2-FOXO-sNPF/NPY Pathway in *Drosophila* and Mammals. PLoS Genet 8, e1002857. doi:10.1371/journal.pgen.1002857

International *Glossina* Genome Initiative, 2014. Genome Sequence of the Tsetse Fly (*Glossina morsitans*): Vector of African Trypanosomiasis. Science 344, 380–386. doi:10.1126/science.1249656

Johard, H.A.D., Enell, L.E., Gustafsson, E., Trifilieff, P., Veenstra, J.A., Nässel, D.R., 2008. Intrinsic neurons of *Drosophila* mushroom bodies express short neuropeptide F: Relations to extrinsic neurons expressing different neurotransmitters. J. Comp. Neurol. 507, 1479–1496. doi:10.1002/cne.21636

Johard, H.A.D., Yoishii, T., Dircksen, H., Cusumano, P., Rouyer, F., Helfrich-Förster, C., Nässel, D.R., 2009. Peptidergic clock neurons in *Drosophila*: Ion transport peptide and short neuropeptide F in subsets of dorsal and ventral lateral neurons. J. Comp. Neurol. 516, 59–73. doi:10.1002/cne.22099

Jones, D.T., 2007. Improving the accuracy of transmembrane protein topology prediction using evolutionary information. Bioinforma. Oxf. Engl. 23, 538–544. doi:10.1093/bioinformatics/btl677

Kahsai, L., Carlsson, M.A., Winther, Å.M.E., Nässel, D.R., 2012. Distribution of metabotropic receptors of serotonin, dopamine, GABA, glutamate, and short neuropeptide F in the central complex of *Drosophila*. Neuroscience 208, 11–26. doi:10.1016/j.neuroscience.2012.02.007

Kahsai, L., Kapan, N., Dircksen, H., Winther, Å.M.E., Nässel, D.R., 2010a. Metabolic Stress Responses in *Drosophila* Are Modulated by Brain Neurosecretory Cells That Produce Multiple Neuropeptides. PLoS ONE 5, e11480. doi:10.1371/journal.pone.0011480

Kahsai, L., Martin, J.-R., Winther, Å.M.E., 2010b. Neuropeptides in the *Drosophila* central complex in modulation of locomotor behavior. J. Exp. Biol. 213, 2256–2265. doi:10.1242/jeb.043190

Kaneko, Y., Hiruma, K., 2014. Short neuropeptide F (sNPF) is a stage-specific suppressor for juvenile hormone biosynthesis by corpora allata, and a critical factor for the initiation of insect metamorphosis. Dev. Biol. 393, 312–319. doi:10.1016/j.ydbio.2014.07.014

Kozak, M., 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. 15, 8125–8148.

Lee, K.-S., Hong, S.-H., Kim, A.-K., Ju, S.-K., Kwon, O.-Y., Yu, K., 2009. Processed short neuropeptide F peptides regulate growth through the ERK-insulin pathway in *Drosophila melanogaster*. FEBS Lett. 583, 2573–2577. doi:10.1016/j.febslet.2009.07.024

Lee, K.-S., Kwon, O.-Y., Lee, J.H., Kwon, K., Min, K.-J., Jung, S.-A., Kim, A.-K., You, K.-H., Tatar, M., Yu, K., 2008. *Drosophila* short neuropeptide F signalling regulates growth by ERK-mediated insulin signalling. Nat Cell Biol 10, 468–475. doi:10.1038/ncb1710

Lee, K.-S., You, K.-H., Choo, J.-K., Han, Y.-M., Yu, K., 2004. *Drosophila* Short Neuropeptide F Regulates Food Intake and Body Size. J. Biol. Chem. 279, 50781–50789. doi:10.1074/jbc.M407842200

Lehane, M.J., 2005. The biology of blood-sucking in insects. Cambridge University Press.

581 Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data
582 Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25,
583 402–408. doi:10.1006/meth.2001.1262
584 Lu, H.-L., Pietrantonio, P., 2011. Immunolocalization of the short neuropeptide F
585 receptor in queen brains and ovaries of the red imported fire ant (*Solenopsis*
586 *invicta Buren*). BMC Neurosci. 12, 1–15. doi:10.1186/1471-2202-12-57
587 Mertens, I., Meeusen, T., Huybrechts, R., De Loof, A., Schoofs, L., 2002.
588 Characterization of the short neuropeptide F receptor from *Drosophila*
589 *melanogaster*. Biochem. Biophys. Res. Commun. 297, 1140–1148.
590 Mikani, A., Wang, Q.-S., Takeda, M., 2012. Brain-midgut short neuropeptide F
591 mechanism that inhibits digestive activity of the American cockroach,
592 *Periplaneta americana* upon starvation. Invertebr. Neuropept. XII 34, 135–144.
593 doi:10.1016/j.peptides.2011.10.028
594 Mikani, A., Watari, Y., Takeda, M., 2015. Brain-midgut cross-talk and autocrine
595 metabolastat via the sNPF/CCAP negative feed-back loop in the American
596 cockroach, *Periplaneta americana*. Cell Tissue Res. 362, 481–496.
597 doi:10.1007/s00441-015-2242-4
598 Nagata, S., Matsumoto, S., Nakane, T., Ohara, A., Morooka, N., Konuma, T., Nagai,
599 C., Nagasawa, H., 2012. Effects of starvation on brain short neuropeptide F-1,
600 -2 and -3 levels and short neuropeptide F receptor expression levels of the
601 silkworm, *Bombyx mori*. Front. Endocrinol. 3. doi:10.3389/fendo.2012.00003
602 Nagata, S., Morooka, N., Matsumoto, S., Kawai, T., Nagasawa, H., 2011. Effects of
603 neuropeptides on feeding initiation in larvae of the silkworm, *Bombyx mori*.
604 CECE 201025th Conf. Eur. Comp. Endocrinol. Conf. Eur. Comp. Endocrinol.
605 172, 90–95. doi:10.1016/j.ygcen.2011.03.004
606 Nässel, D.R., Enell, L.E., Santos, J.G., Wegener, C., Johard, H.A., 2008. A large
607 population of diverse neurons in the *Drosophila* central nervous system
608 expresses short neuropeptide F, suggesting multiple distributed peptide
609 functions. BMC Neurosci. 9, 90–90. doi:10.1186/1471-2202-9-90
610 Nässel, D.R., Wegener, C., 2011. A comparative review of short and long
611 neuropeptide F signaling in invertebrates: Any similarities to vertebrate
612 neuropeptide Y signaling? Peptides 32, 1335–1355.
613 doi:10.1016/j.peptides.2011.03.013
614 Offermanns, S., Simon, M.I., 1995. Gα15 and Gα16 Couple a Wide Variety of
615 Receptors to Phospholipase C. J. Biol. Chem. 270, 15175–15180.
616 doi:10.1074/jbc.270.25.15175
617 Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0:
618 discriminating signal peptides from transmembrane regions. Nat Meth 8, 785–
619 786. doi:10.1038/nmeth.1701
620 Predel, R., Neupert, S., Garczynski, S.F., Crim, J.W., Brown, M.R., Russell, W.K.,
621 Kahnt, J., Russell, D.H., Nachman, R.J., 2010. Neuropeptidomics of the
622 Mosquito *Aedes aegypti*. J. Proteome Res. 9, 2006–2015.
623 doi:10.1021/pr901187p
624 Ren, J., Wen, L., Gao, X., Jin, C., Xue, Y., Yao, X., 2008. CSS-Palm 2.0: an updated
625 software for palmitoylation sites prediction. Protein Eng. Des. Sel. 21, 639–
626 644. doi:10.1093/protein/gzn039
627 Root, C.M., Ko, K.I., Jafari, A., Wang, J.W., 2011. Presynaptic Facilitation by
628 Neuropeptide Signaling Mediates Odor-Driven Food Search. Cell 145, 133–
629 144. doi:10.1016/j.cell.2011.02.008

630 Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R.,
631 McWilliam, H., Remmert, M., Söding, J., Thompson, J.D., Higgins, D.G., 2011.
632 Fast, scalable generation of high-quality protein multiple sequence alignments
633 using Clustal Omega. Mol. Syst. Biol. 7. doi:10.1038/msb.2011.75
634 Spit, J., Badisco, L., Verlinden, H., Van Wielendaele, P., Zels, S., Dillen, S., Vanden
635 Broeck, J., 2012. Peptidergic control of food intake and digestion in insects
636 Can. J. Zool. 90, 489–506. doi:10.1139/z2012-014
637 Stables, J., Green, a, Marshall, F., Fraser, N., Knight, E., Sautel, M., Milligan, G.,
638 Lee, M., Rees, S., 1997. A bioluminescent assay for agonist activity at
639 potentially any G-protein-coupled receptor. Anal. Biochem.
640 doi:10.1006/abio.1997.2308
641 Tobe, S.S., Langley, P.A., 1978. Reproductive Physiology of Glossina. Annu. Rev.
642 Entomol. 23, 283–307. doi:10.1146/annurev.en.23.010178.001435
643 Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A.,
644 Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR
645 data by geometric averaging of multiple internal control genes. Genome Biol.
646 3, research0034.1–research0034.11.
647 Veenstra, J.A., 2000. Mono- and dibasic proteolytic cleavage sites in insect
648 neuroendocrine peptide precursors. Arch. Insect Biochem. Physiol. 43, 49–63.
649 doi:10.1002/(SICI)1520-6327(200002)43:2<49::AID-ARCH1>3.0.CO;2-M
650 Veenstra, J.A., Rombauts, S., Grbić, M., 2012. In silico cloning of genes encoding
651 neuropeptides, neurohormones and their putative G-protein coupled receptors
652 in a spider mite. Insect Biochem. Mol. Biol. 42, 277–295.
653 doi:10.1016/j.ibmb.2011.12.009
654 Yamanaka, N., Yamamoto, S., Žitňan, D., Watanabe, K., Kawada, T., Satake, H.,
655 Kaneko, Y., Hiruma, K., Tanaka, Y., Shinoda, T., Kataoka, H., 2008.
656 Neuropeptide Receptor Transcriptome Reveals Unidentified Neuroendocrine
657 Pathways. PLoS ONE 3, e3048. doi:10.1371/journal.pone.0003048
658

Figure 1: Cloned *Glomo-snpf* cDNA sequence with corresponding prepropeptide sequence. A predicted signal peptide is indicated in bold and italics. Intron positions are represented by blue boxes. The Glomo-sNPF sequences are indicated in black with conserved residues of the insect sNPF consensus sequence underlined: [xPxLRLRFamide] (Nässel and Wegener, 2011). Dibasic and monobasic cleaving sites are shown in gray. Monobasic cleavage sites consist of KX_nR or RX_nR where n = 2,4,6 or 8 and cleavage occurs after X_n (Veenstra, 2000).

Figure 2: *G. m. morsitans* sNPFR sequence with predicted transmembrane domains (underlined), rhodopsin-like GPCR motifs (bold) and sites for post-translational modifications (coloured). The typical rhodopsin-like GPCR motifs consist of: GN in helix 1, NLX_3DX_8P in helix 2, $SX_3LX_2IX_2DRY$ in helix 3, WX_8P in helix 4, FX_2PX_7Y in helix 5, FX_3WXP in helix 6 and NPX_2YX_6F in helix 7. Post-translational modifications in yellow: predicted glycosylation sites in the extracellular loops or N-terminal tail, in green: putative phosphorylation sites in the intracellular loops or C-terminal tail, in red: putative palmitoylation site in the C-terminal tail.

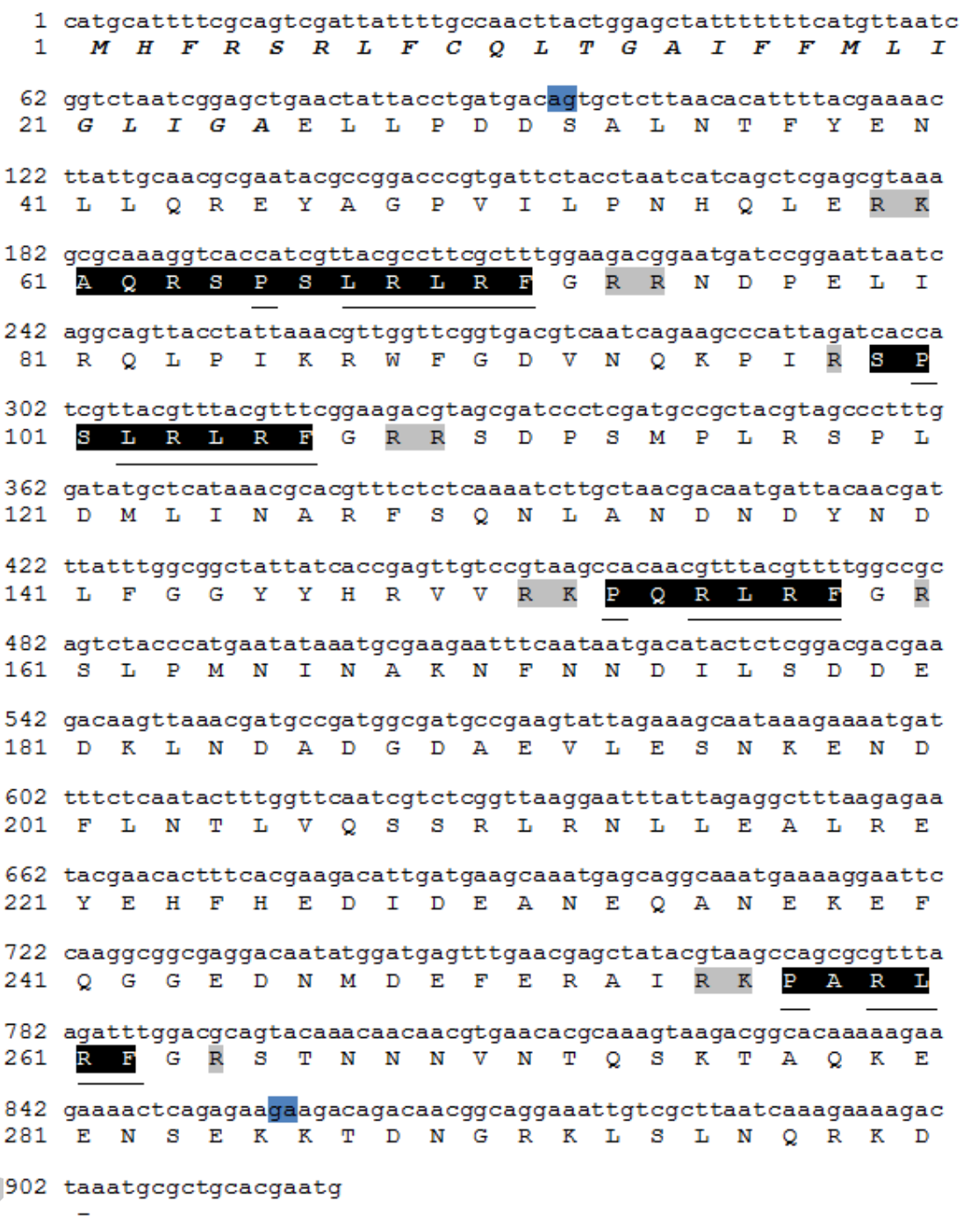
Figure 3: Expression profiles of (A) *Glomo-snpfr* and (B) *Glomo-snpf* relative to transcript levels in the female head (He) in tissues collected from adult female flies or mixed larval instars (no differentiation in sex could be made for larvae in different instars). The mean value of three biological replicates measured in triplicate is presented. Error bars indicate SEM. CC-CA: corpora cardiaca – corpora allata; TG: thoracic ganglion; SG: salivary gland; MT: Malpighian tubules; AM: anterior midgut; PM: posterior midgut; HG: hindgut; FB: fat body; RS: reproductive system; Mu: flight muscles; Br: brain; He: head; Pu-18: 18-day-old pupa; Th: thorax; Ab: abdomen; In: instar; Pu-11: 11-day-old pupa.

Figure 4: Calcium response curves of CHO/mtAeq/$G\alpha_{16}$ cells expressing Glomo-sNPFR challenged with concentration series of the Glomo-sNPF peptides. Mean values, corrected for the negative control BSA, of three independent measurements consisting of three replicates of the concentration series are represented with corresponding ± SEM. The EC_{50} value is depicted with its 95% confidence interval.

Table 1: EC_{50} values of the calcium mobilization assay performed in transiently transfected CHO/mtAeq cells.

Peptide	Sequence	Receptor	EC_{50} value
Glomo-sNPF-1	AQRSPSLRLRFa	Glomo-sNPFR	2.21 ± 1.87 nM
Glomo-sNPF-2	SPSLRLRFa	Glomo-sNPFR	3.42 ± 2.99 nM
Glomo-sNPF-3	PQRLRFa	Glomo-sNPFR	2.67 ± 2.27 nM
Glomo-sNPF-4	PARLRFa	Glomo-sNPFR	11.83 ± 9.25 nM

691



692

693

694 Fig. 1

695

696

697

698

699 Fig. 2

700

701

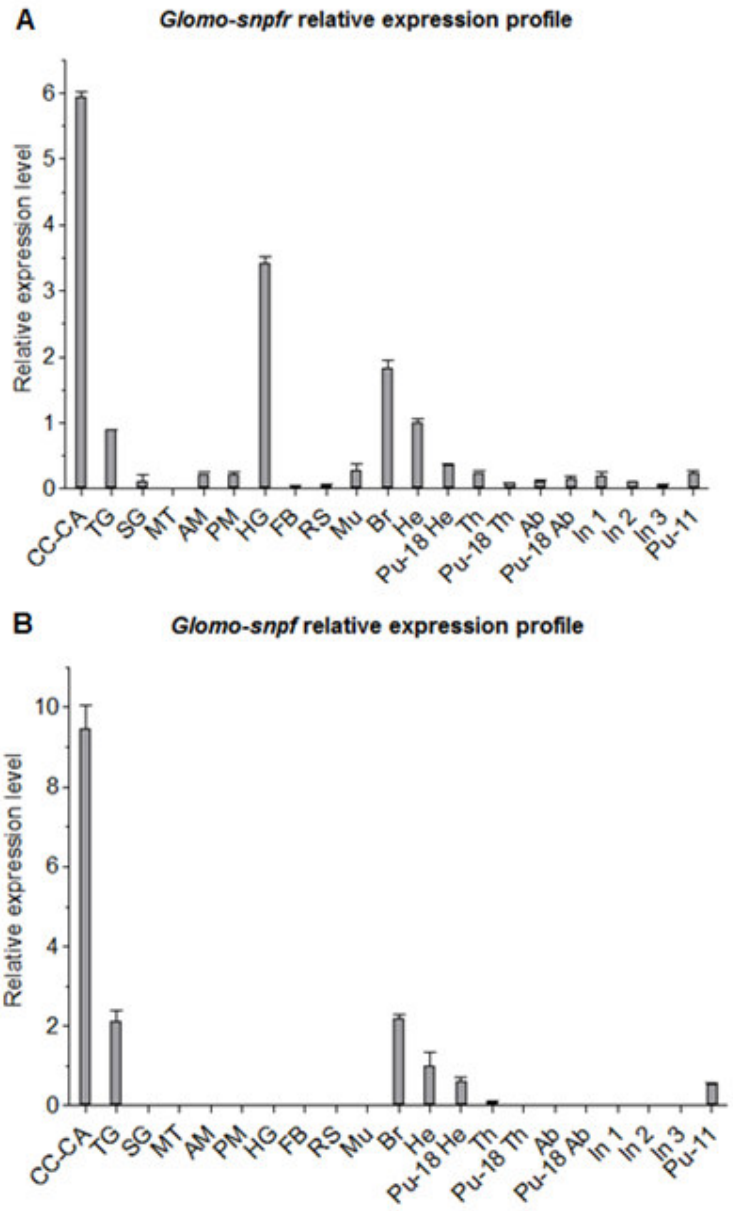
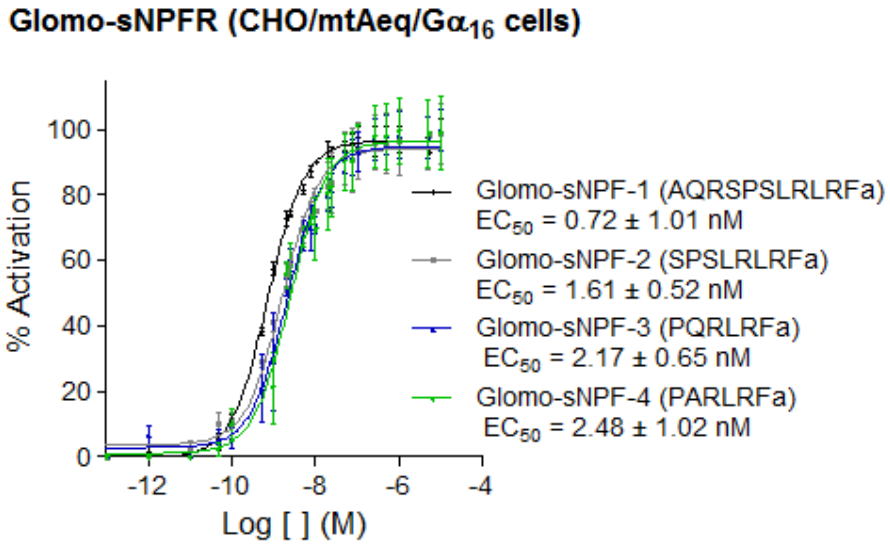

A
Glomo-snpfr relative expression profile
Relative expression level
0
1
2
3
4
5
6
CC-CA
TG
SG
MT
AM
PM
HG
FB
RS
Mu
Br
He
Pu-18 He
Th
Pu-18 Th
Ab
Pu-18 Ab
In 1
In 2
In 3
Pu-11
B
Glomo-snpf relative expression profile
Relative expression level
0
2
4
6
8
10
CC-CA
TG
SG
MT
AM
PM
HG
FB
RS
Mu
Br
He
Pu-18 He
Th
Pu-18 Th
Ab
Pu-18 Ab
In 1
In 2
In 3
Pu-11

Fig. 3



707

708

709 Fig. 4

710

711

712 Highlights

713 Cloning of *G. m. morsitans snpfr* and s*npf* cDNA sequences confirms the presence of
714 sNPF signaling components in tsetse flies.

715 Four Glomo-sNPF peptides concentration-dependently activate Glomo-sNPFR in
716 heterologous cells.

717 *Glomo-snpfr* transcripts in female tsetse flies are exclusively expressed at adult stage
718 and predominantly found in the corpora cardiaca-corpora allata and the hindgut.

719 *Glomo-snpf* transcripts are similarly expressed in the central nervous system of adult
720 females and additionally in pupal developmentpupae.

721

722